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We previously demonstrated elevated HGF and Met expression in regions of invasive human breast carcinomas, suggesting a role of autocrine HGF loops in invasive breast cancer. We have now demonstrated that: a) elevated HGF and Met expression occurs in some newly-derived breast and non-small cell lung carcinoma cell lines; and b) three carcinoma cell lines showed autocrine tyrosine-phosphorylation of Met and increased cell survival, consistent with the presence of autocrine HGF loops. These findings suggest that targeting autocrine HGF loops is an important strategy for therapeutic intervention in breast cancer metastasis. Based on 3-D computer modelling of the Met receptor binding site in the K1 kringle domain of HGF, we have initiated a phage display approach to screen 7-10 mer peptide libraries for putative peptide antagonists of HGF that block ligand receptor binding. These putative peptide antagonists will be isolated, and tested for the ability to inhibit tumorigenesis and metastasis of breast carcinoma cells.

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PREFACE:

Please note that the principal investigator of this award, Jin Gui, withdrew from the Ph.D. programme in May, 2000 for academic reasons, following a recommendation from the School of Graduate Studies at Queen's University. During the first year of his programme, experiments were performed relating to Objective I, by Jin Gui and by other members of the laboratory (through collaboration, see App. I). The USAMRMC office has been informed of this situation, and we are in the progress of recruiting a new student to continue this project.

INTRODUCTION:

Role of HGF in mammary tumor progression: Expression of HGF in nonmalignant epithelium is generally under tight negative regulation (1). Paracrine stimulation of normal breast epithelium with HGF, in co-operation with other growth factors (e.g., neuregulin), promotes branching morphogenesis (2). However, HGF (3-5) and its receptor, Met (6), are frequently over-expressed in breast cancer as well as in many other cancer types. This high level of HGF and Met expression has been identified as a possible independent predictor of poor survival in breast cancer patients (6). Recent findings from our laboratory show that invasive human carcinoma cells co-express HGF and Met, particularly at the migrating tumor front (5). Furthermore, expression of HGF or a constitutively active mutant form of Met (Tpr-Met) in transgenic mice (7,8,9) or in transformed cell lines (10,11,12) promotes tumorigenesis and metastasis. During tumorigenesis, HGF stimulates loss of epithelial differentiation (13), increased cell survival (14,15), invasion (16), and metastasis (10; 17). These observations have suggested that sustained activation of the Met signal transduction pathway may be important for development of cancers through autocrine or paracrine mechanisms.

Structure, maturation and isoforms of HGF: Mature HGF protein contains a heparin binding domain, followed by four kringle-like domains designated K1 to K4, as well as a C-terminal region homologous to serine protease but lacking any protease activity (18). In human, HGF is first produced as a single polypeptide precursor of 728 amino acids. Following removal of the N-terminal 31 amino acids, this pro-hormone form is secreted and then cleaved by an unknown serine protease to form the active mature HGF (19). The cleavage at the Arg^{494}-Val^{495} site is essential for HGF activity and several serine proteases have been shown to process pro-HGF at this site *in vitro* (20). Other naturally occurring forms of HGF are also found *in vivo*. Truncated versions of HGF derived from alternative mRNA splicing can act either antagonistically or agonistically towards native HGF. NK1 which contains all the N-terminal amino acids up to K1 can bind to Met and acts in both agonistic or antagonistic manners to HGF depending on culture conditions (21-23). NK2, containing the HGF sequence up to K2, acts mainly in an antagonistic manner (24). The presence of these isoforms in nature may provide a subtle way of regulating HGF activity *in vivo*.

HYPOTHESIS:

Whereas nonmalignant epithelial cells do not express HGF, we have shown abnormal co-expression of HGF and Met in human breast carcinomas (5) and in carcinoma cell lines. Based on these findings, our hypothesis is that **co-expression of HGF and Met results in the**

establishment of an HGF autocrine loop which provides a selective advantage for autonomous growth and metastasis of mammary carcinoma cells. The following predictions can be made from this hypothesis: Breast carcinoma cells should express increased levels of HGF and Met compared to nonmalignant epithelial cells; *in vivo* tumor microenvironment may be important in sustaining high levels of HGF and Met expression. Inhibition of an existing HGF autocrine loop by blocking the interaction of HGF and Met would be expected to abrogate HGF-dependent functions and to suppress tumorigenic properties.

The following OBJECTIVES are proposed:

I): To assess the expression and function of HGF and Met in nonmalignant and malignant breast epithelial cells, and to correlate with HGF-induced cellular functions;

II): To design peptide antagonists that block the interaction between HGF and Met, and to determine the effect of interruption of paracrine and autocrine HGF loops on growth and metastasis of breast carcinomas.

MATERIALS AND METHODS: Breast carcinoma cell lines used in this study are described in Table I. Details of materials and methods are described in App. I and Figure legends.

PROGRESS AND RESULTS:

Objective (I): To assess the expression and function of HGF and Met in nonmalignant and malignant breast epithelial cells, and to correlate with HGF-induced cellular functions:

Using *in situ* hybridization, we (5) and other laboratories (25,26) have shown that HGF mRNA is expressed in human invasive breast carcinoma cells as well as in regions of ductal epithelial hyperplasia. Preliminary results also showed immunoreactive HGF protein associated with carcinoma cells in human breast tissues (Tuck *et al.*, unpublished result). However, it is not known from these studies whether post-translational processing of pro-HGF, required for HGF activity, occurs in benign or malignant epithelial cells from primary breast tissues. Since transcriptional regulation of HGF in normal epithelium is under tight negative regulation (1), it was important to determine whether functionally active HGF protein was produced by malignant carcinoma cells. We therefore examined the expression and activity of HGF and Met in newly-established nonmalignant and malignant breast epithelial cell lines.

a) Detection of HGF mRNA and protein: To detect expression of HGF mRNA in nonmalignant and malignant breast epithelial cells, we used RT-PCR analysis. Primers were designed to detect HGF cDNA, and to recognize the corresponding cDNAs of mouse and human but not the homologous family members MSP (Fig. 1). Primers specific for the house-keeping gene, β-glucuronidase (GUS B (31)), were used as an internal control. A linear range of amplification was found between 10 and 30 cycles for HGF and GUS B. Similarly, the product amplification was

directly proportional to the amount of template used (data not shown).

To aid the studies of HGF protein, we used a copper (II) affinity chromatography technique to isolate HGF from conditioned media of cell lines, developed in my laboratory (32). The principal of separation of HGF from biological samples by Cu (II) affinity chromatography is based on the fact that HGF has several cationic sequences (His-X-His) in the kringle domains of the HGF molecule. Greater than 70% of the HGF protein bound to the copper (II) column was eluted with 80 mM imidazole, as determined by western blotting.

b) Expression of HGF and Met mRNA and protein in newly established human breast carcinoma and non-small cell lung carcinoma cell lines: Our results showed that 12/12 mammary epithelial and carcinoma cell lines expressed Met mRNA, and all but one carcinoma cell line (WO-E) expressed Met protein (Table I, Ref. 28-30). In addition, HGF mRNA was expressed in 8/12 mammary epithelial and carcinoma cell lines tested (Fig. 1C), and five cell lines tested expressed HGF protein, detected by western blotting (Fig. 2A & Table I). One breast carcinoma cell line, MCF10A1T3B, showed several immunoreactive lower molecular weight bands, which were not present in the corresponding non-malignant cell line MCF10A1 or another carcinoma cell line, EL-E. The possible function of the observed lower molecular weight bands in CM from MCF10A1T3B cells is currently being examined (see below).

Recently, non-small cell lung carcinomas (NSCLC) have been shown to express HGF mRNA and protein (41). Therefore, to provide a comparison with another carcinoma cell type, we have also examined HGF and Met expression in a series of NSCLC cell lines. Our results, summarized in Table II, showed that 2/6 NSCLC cell lines expressed Met protein. In addition, 4/6 NSCLC cell lines expressed HGF mRNA and protein (Fig. 3A), two of which also expressed Met. One nonmalignant human bronchioepithelial cell line (HBE) (34) showed no detectable PCR product for HGF and was used as a baseline control for comparison with other cell lines (App. I and Table II).

c) Activity of putative HGF protein secreted by mammary and NSCLC carcinoma cell lines: To assess the activity of HGF in CM from carcinoma cell lines, we tested the ability of CMs to activate Met in A549 carcinoma cells (which are Met positive, HGF negative). A549 cells were incubated for 30 min at 37°C with CMs, and lysed. Cell lysates were immunoprecipitated with anti-Met IgG, and subjected to SDS-PAGE (reducing conditions) and western blotting with anti-phosphotyrosine antibody to assess the tyrosine phosphorylation status of Met (Fig. 2B). A control showed strong tyrosine phosphorylation of Met in A549 cells incubated with HGF (40 ng/ml), compared to A549 cells incubated alone. The results showed that CM from EL-E, MCF10A1 and MCF10A1T3B cell lines stimulated tyrosine-phosphorylation of Met in A549 cells. In addition, 3/4 HGF-producing NSCLC cell lines showed activity in the Met-phosphorylation assay (Fig. 3B). Interestingly, CM collected from MCF10A1T3 B cells induces tyrosine-phosphorylation of Met in A549 cells to a lesser extent than CM from EL-E cells, suggesting that there is less active HGF in MCF10A1T3B CM. In contrast, CM from WO-E cells, which showed no HGF protein, had no effect. These results indicate that the majority of breast carcinoma and NSCLC cell lines secrete active HGF (See Table II and App. I).

d) Examination of Met activation status in breast carcinoma and NSCLC cell lines: A prediction from the above studies is that carcinoma cell lines that express both active HGF and Met would show constitutive activation of Met, consistent with the establishment of an autocrine HGF loop in these cells. To test this possibility, we have examined the tyrosine-phosphorylation level of Met in carcinoma cell lines, using western blot analysis. One mouse carcinoma cell line showed co-expression of HGF and tyrosine-phosphorylated Met, and spontaneous invasion through matrigel (27). None of the human breast carcinoma cell lines tested showed autocrine tyrosine-phosphorylation of Met, although addition of exogenous HGF stimulated tyrosine-phosphorylation of Met in all cases (data not shown). However, the possibility that autocrine activity of HGF is inhibited by ligand degradation, or association with proteoglycans (e.g. heparin) (42), is currently being examined.

Interestingly, two NSCLC cell lines (WT-E and SW900) expressing both HGF and Met showed significant tyrosine-phosphorylation of Met even without treatment with exogenous HGF (Fig. 4). These experiments are consistent with establishment of an autocrine HGF loop in some carcinoma cells co-expressing HGF and Met.

e) DNA synthesis and survival in carcinoma cell lines: To test the biological function of putative paracrine versus autocrine activation of Met in NSCLC cell lines, we examined DNA synthesis and cell survival in various NSCLC cell lines. The base level of DNA synthesis in the absence of exogenous HGF was low in all cell lines, regardless of the level of endogenous HGF produced (Fig. 5C). All Met-positive cell lines required paracrine stimulation with HGF of DNA synthesis, regardless of the level of endogenous HGF produced. A corresponding HGF-dependent increase in ERK1/2 activation was also detected (Fig. 5D). Cell lines which showed no expression of Met did not respond to HGF in the DNA synthesis assay (data not shown).

In contrast, cell lines (SW-900 and WT-E) with autocrine expression of HGF and sustained tyrosine-phosphorylation of Met, consistently showed increased survival under nonadherent serum-starved conditions; whereas A549 cells, which express Met but not HGF, showed a reduced survival response (Fig. 5A). Furthermore, SW-900 and WT-E cells showed sustained high level of tyrosine-phosphorylation of Met under nonadherent conditions compared to A549 cells which did not (Fig. 5B). Thus increased cell survival, but not DNA synthesis, correlated with the expression of HGF and autophosphorylation of Met at tyrosine residues in NSCLC cell lines.

Objective (II): To design peptide antagonists that block the interaction between HGF and Met, and to determine the effect of interruption of paracrine and autocrine HGF loops on growth and metastasis of breast carcinomas.

It is anticipated that combined strategies may be required to inhibit HGF functions in breast tumor cells. Therefore, in addition to testing antisense oligonucleotides, we have initiated a new approach to isolate short peptide antagonists of HGF using a technique referred to as "phage display" to screen random peptide libraries (36). This approach allows the rapid identification of short peptides which bind specifically to certain growth factors or their receptors, block binding of the growth factor to its receptor, and inhibit growth factor function (37-39). These peptide "antagonists" may pave the way for a new generation of potent anti-cancer agents which can be delivered with high efficiency to the tumor site. As a first step, we have used 3-D computer modelling of the Met-binding site in the K1 kringle domain (Fig. 6), based on previously reported site-specific mutational studies

and x-ray crystallography of the K1 domain (40). The results suggest the presence of a groove of approximately 7-10 a.a. in length, corresponding to the Met binding site. Based on this information we have chosen heptapetide and decapeptide libraries for screening of libraries. HGF-binding phage will be selected from phage-display libraries using a panning procedure with recombinant NK1 peptide as absorbent. Screening of selected phage will be carried out using a high-throughput ELISA technique (36). Positive NK1-binding phage will be further characterized as possible candidates for HGF antagonism. I have recently received funds from the Canadian Breast Cancer Research Initiative to continue this study.

KEY RESEARCH ACCOMPLISHMENTS:

During this grant period, we have made the following observations:

- Elevated HGF and Met expression occurs in some newly-derived breast and non-small cell lung carcinoma cell lines.

- Several carcinoma cell lines showed autocrine tyrosine-phosphorylation of Met and increased cell survival, consistent with the presence of autocrine HGF loops in some breast and lung carcinomas.

- Using 3-D computer modelling of the K1 kringle domain, we have predicted the presence of a groove of approximately 7-10 a.a. in length, corresponding to the Met binding site.

- Information from these studies could lead to novel therapeutic approaches to breast cancer.

REPORTABLE OUTCOMES:

The following manuscript in preparation has developed from this study:

"Identification of paracrine and possible autocrine hepatocyte growth factor loops in non-small cell lung carcinomas." Qiao, H., J. Gui, W. Hung, E. Tremblay, J. Ho, J. Klassen, B. Campling, R. Schwall and B.E. Elliott. Manuscript in preparation.

CONCLUSIONS:

We previously demonstrated elevated HGF and Met expression in regions of invasive human breast carcinomas, suggesting a role of autocrine HGF loops in invasive breast cancer. We have now demonstrated that: a) elevated HGF and Met expression occurs in some newly-derived breast and non-small cell lung carcinoma cell lines; b) three carcinoma cell lines showed autocrine tyrosine-phosphorylation of Met and increased cell survival, consistent with the presence of autocrine

HGF loops; and c) the K1 kringle domain of HGF contains a groove of approximately 7-10 a.a. in length, corresponding to the Met binding site, as determined using 3-D computer modelling. These observation are further discussed below.

a) Our demonstration of HGF expression in many breast carcinoma cell lines validates our earlier observation using *in situ* hybridization that HGF and Met are co-expressed in carcinoma cells in regions of invasive breast cancer. Similarly, several breast carcinoma and NSCLC cell lines showed increased HGF expression. Three cell lines (SP1, SW-900 and WT-E) showed increased tyrosine phosphorylation of Met. Several cell lines showed no phosphorylation of Met although significant HGF activity was detected in conditioned media from these cell lines. Thus, HGF secreted by these cells was either insufficient or ineffective in activating Met. In one breast carcinoma cell line, MCF10A1T3B, two smaller molecular weight forms of HGF were detected, in addition to pro-HGF and mature HGF proteins, suggesting the presence of degraded native HGF, or HGF isoforms (Fig. 2A). Production of naturally occurring isoforms, such as NK2 (Mr-30 kDa) which can act as an antagonist in some systems (21-23), or proteolytic degradation may have an inhibitory effect on activity of HGF produced by such carcinoma cells. Our preliminary studies (data not shown) suggest that MCF10A1T3B cells express proteases that degrade secreted HGF. Experiments involving protease inhibitors are in progress to confirm these findings.

b) Two NSCLC cell lines (SW-900 and WT-E) which express high levels of Met and secreted HGF protein, showed tyrosine-phosphorylation of Met, consistent with an autocrine HGF loop. To determine the functional relevance of the elevated Met activation in these cells, we examined DNA synthesis and cell survival. All Met-expressing NSCLC cell lines required paracrine stimulation with HGF for an optimal proliferation response, regardless of the presence of an autocrine HGF loop. In contrast, two NSCLC cell lines (SW-900 and WT-E), which express an autocrine HGF loop, showed a sustained high level of Met tyrosine-phosphorylation and cell survival under nonadherent conditions. In an independent study, Yi *et al.*, (41) showed that NSCLC cell lines expressing HGF mRNA exhibited paracrine, but not autocrine, stimulation by HGF of DNA synthesis; however the level of constitutive activation of Met was not assessed in their study. Our results indicate that autocrine activation of Met is sufficient to stimulate cell survival, whereas additional paracrine stimulation with HGF is required to stimulate DNA synthesis in NSCLC cells. Experiments are in progress to further assess the pattern of HGF-induced signalling and functions associated with paracrine versus autocrine HGF stimulation of carcinoma cells.

c) Overall, we have identified autocrine HGF loops in breast carcinoma and NSCLC cell lines, and have correlated the activation of autocrine HGF loops with increased cell survival. These findings suggest that targeting autocrine HGF loops is an important strategy for therapeutic intervention in breast cancer metastasis. Based on 3-D computer modelling of the Met receptor binding site in the K1 kringle domain of HGF, we have initiated a phage display approach to screen 7-10 mer peptide libraries for putative peptide antagonists of HGF that block ligand receptor binding. In the next stage of this work, these putative peptide antagonists will isolated, and tested for the ability to inhibit tumorigenesis and metastasis of breast carcinoma cells.

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TABLE I

Expression of HGF and Met in human breast epithelial and carcinoma cell lines

Breast Cell Line	Species	Malignant Status	HGF			Met	
			mRNA	Protein	Activity	mRNA	Protein
WO-E [a]	Human	Yes	−[f]	−	-	+	−
EL-E [a]	Human	Yes	+	+	+	+	+
HU-E [a]	Human	Yes	ND	ND	ND	ND	ND
MCF10A1 [b]	Human	No	trace	+	trace	+	+
MCF10A1T3B [b]	Human	Yes	trace	+	trace	+	+
21PT [c]	Human	No	trace	ND	−	+	+
21NT [c]	Human	Yes	trace	ND	−	+	+
21MT-1 [c]	Human	Yes	trace	ND	−	+	+
TM3 [d]	Mouse	No	ND	−	-	ND	+
T-2410L TM6 [d]	Mouse	Yes	ND	−	-	ND	+
SP1 [e]	Mouse	Yes	+	+	+	+	+
SP1- 3M [e]	Mouse	Yes	+	+	+	+	+

Table I Legend:

a) EL-E, WO-E and HU-E are human breast carcinoma cell lines derived from human breast cancer patients (obtained from Dr. B. Campling, Cancer Research Lab., Queen's University).

b) MCF10A1 is a subclone of a spontaneously immortalized non-tumorigenic human breast epithelial cell line established from long term culture of a breast subcutaneous mastectomy. MCF10A1T3B is a cell line derived from *Ha-Ras* transfected MCF10A1 cells growing as a tumor in a nude mouse (obtained from Dr. F. Miller, Michigan Cancer Foundation).

c) Cell lines were derived from a patient with infiltrating breast carcinoma (obtained from Dr. R. Sager, Dana Farber Cancer Institute).

d) TM3 is a Balb/c mouse-derived mammary epithelial cell line (obtained from D. Medina, Baylor College). T-2410LTM6 is a carcinoma cell line derived from TM3 cell.

e) SP1 is a murine mammary carcinoma which arose spontaneously in a CBA female mouse. SP1-3M is a highly metastatic variant subclone of SP1 selected by serial passage of a metastatic nodule into the mammary fat pad.

f) ND, not determined; +, positive; -, negative.

TABLE II

Expression of HGF and Met in human Non-small cell lung carcinoma cell lines

Carcinoma Cell Lines	Histology	Origin	HGF			Met		HGF-induced DNA synthesis[c]
			mRNA	Protein	Activity	mRNA	Protein	
SW-900	squamous cell carcinoma	primary tumor	+[b]	+	+	+	+	+
WT-E	squamous cell carcinoma	pleural effusion	trace	+	-	+	+	+
SK-Luci-6	large cell anaplastic	primary tumor	+	+	+	+	-	-
QU-DB	large cell anaplastic	primary tumor	trace	-	-	+	-	-
BH-E	adenocarcinoma	pleural effusion	+	+	+	+	-	-
LC-T	adenocarcinoma	primary tumor	-	-	-	+	-	-

Legend:

a) See Materials and Methods for designation of Non-small cell lung carcinoma cell lines.

b) +, positive; -, negative.

c) Cells (1×10^4) in triplicate were incubated in a 24 well plate for 24 hours at 37°C and 5% CO_2, alone, or with HGF (20 ng/ml). After 24 h, ^{3}H-Thymidine was added, and cells were incubated for a second 24 h period. DNA synthesis was measured as incorporation of ^{3}H-Thymidine.

Figure 1

A

PCR product for HGF mRNA

SP HP K1 K2 K3 K4 Protease-like Domain

B

← 206
← 560
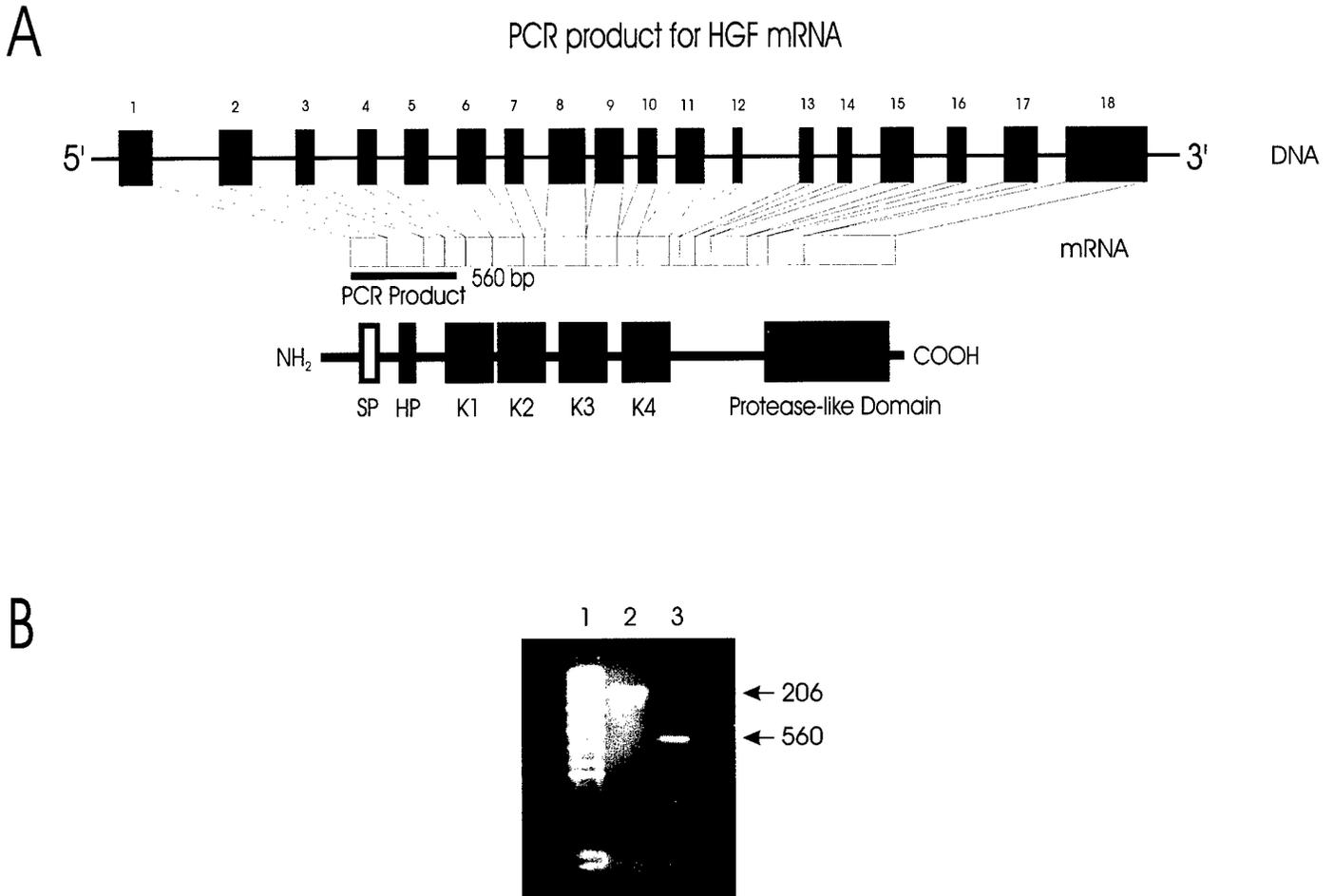
Design of PCR primers for detection of HGF mRNA

Panel A) Primers were designed to overlap more than one exon and to crossreact between mouse and human HGF primers: 5' (sense) TGT CGC CAT CCC CTA TGC AG   (corresp. to bases 69-88 of hHGF); 3' (antisense) TCA ACT TCT GAA CAC TGA GG. (corresp. to bases 610-629 of hHGF). Panel B) cDNA was prepared from 1 µg of total RNA, and subjected to RT-PCR of 25 cycles of: 1 min at 95°C (denaturing), 1 min at 55°C (annealing), and 1 min at 72°C (elongation). Lane 1: DNA molecular size markers; lane 2, 206 bp marker; lane 3, PCR product of HGF.

Figure 1

C
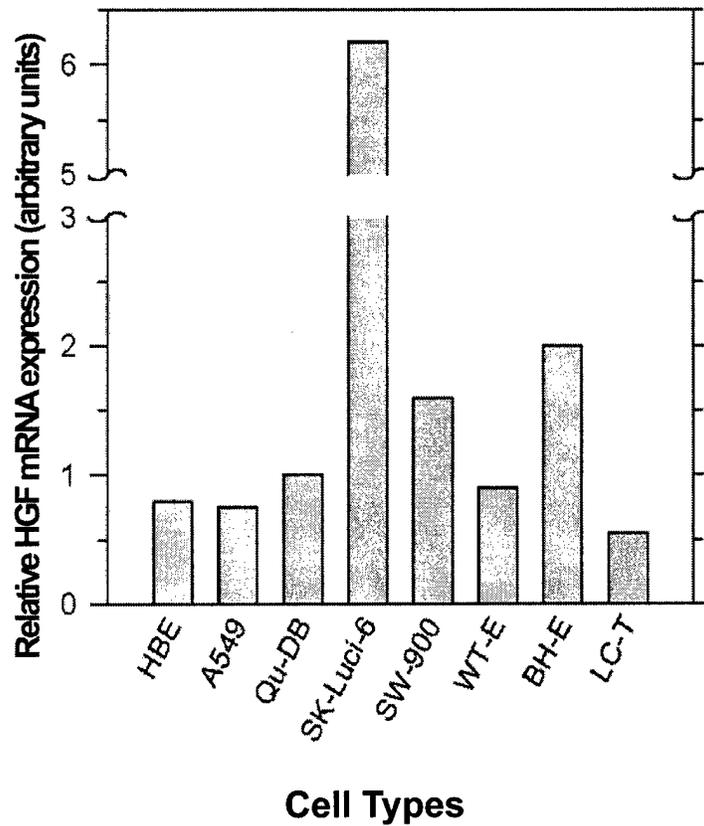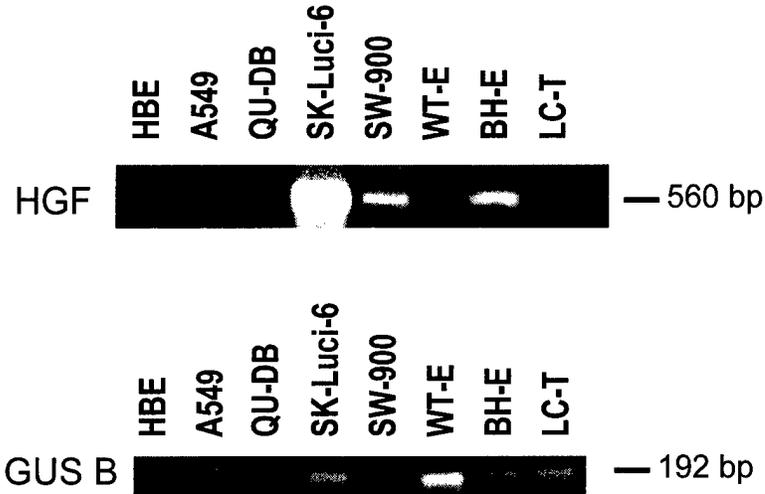
HGF — 560 bp

(lanes: HBE, A549, QU-DB, SK-Luci-6, SW-900, WT-E, BH-E, LC-T)

GUS B — 192 bp

(lanes: HBE, A549, QU-DB, SK-Luci-6, SW-900, WT-E, BH-E, LC-T)

Relative HGF mRNA expression (arbitrary units) vs Cell Types (HBE, A549, Qu-DB, SK-Luci-6, SW-900, WT-E, BH-E, LC-T)

Analysis of NSCLC cell lines

Total RNA from various NSCLC cell lines was extracted and used in a reverse transcription reaction to produce cDNA. A nonmalignant lung bronchial cell line, HBE (34), was used as a control. Primers specific for HGF and GUS B (as internal control) were added to the cDNA. Unlabelled (upper) and labelled (lower) PCR was carried out with 25 cycles, and the reaction products were analysed as described in App.I.

# Figure 2

A

B
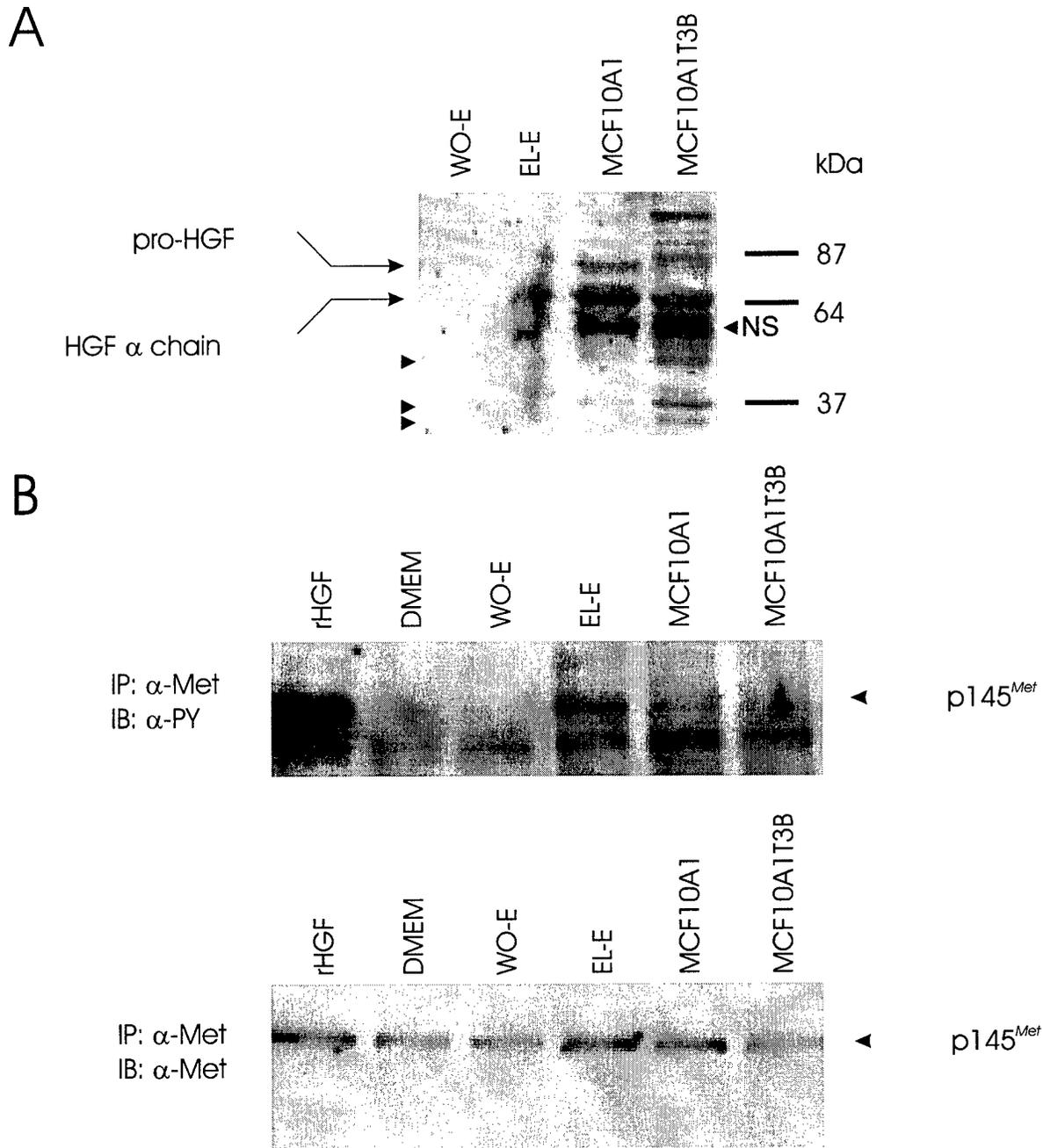
Expression and activity of HGF secreted by human breast carcinoma cell lines

Panel A) Conditioned media (CM) from newly established human breast carcinoma cell lines were subjected to Cu(II)-affinity chromatography (42), and eluted proteins were subjected to SDS-PAGE under reducing conditions. Western blotting with anti-HGF IgG revealed bands corresponding to pro-HGF and mature HGF α-chain. Arrows indicates putative degradation fragments and/or isoforms of HGF. A non-specific band (NS) at 55 kDa was present in most lanes. Panel B) CMs from cell lines in A) were tested for HGF activity as determined by the ability to stimulate tyrosine-phosphorylation of Met in A549 cells, which express Met, but not HGF. CM from EL-E showed stronger activity of HGF, than from MCF10A1T3B. See Table I.

Figure 3

A

Detection of HGF protein in conditioned media of NSCLC cell lines

The presence of HGF protein in the conditioned media collected from different lung carcinoma cell lines was determined using copper (II) affinity chromatography to purify putative HGF from conditioned media. Fractions containing putative HGF were concentrated by Microcon concentrators, and were analysed by reducing SDS-PAGE, followed by western blotting with sheep anti-HGF antibody (Genentech). Immunoreactive bands were revealed by ECL. Arrows corresponding to pro-HGF and mature HGF are shown.

Figure 3

B
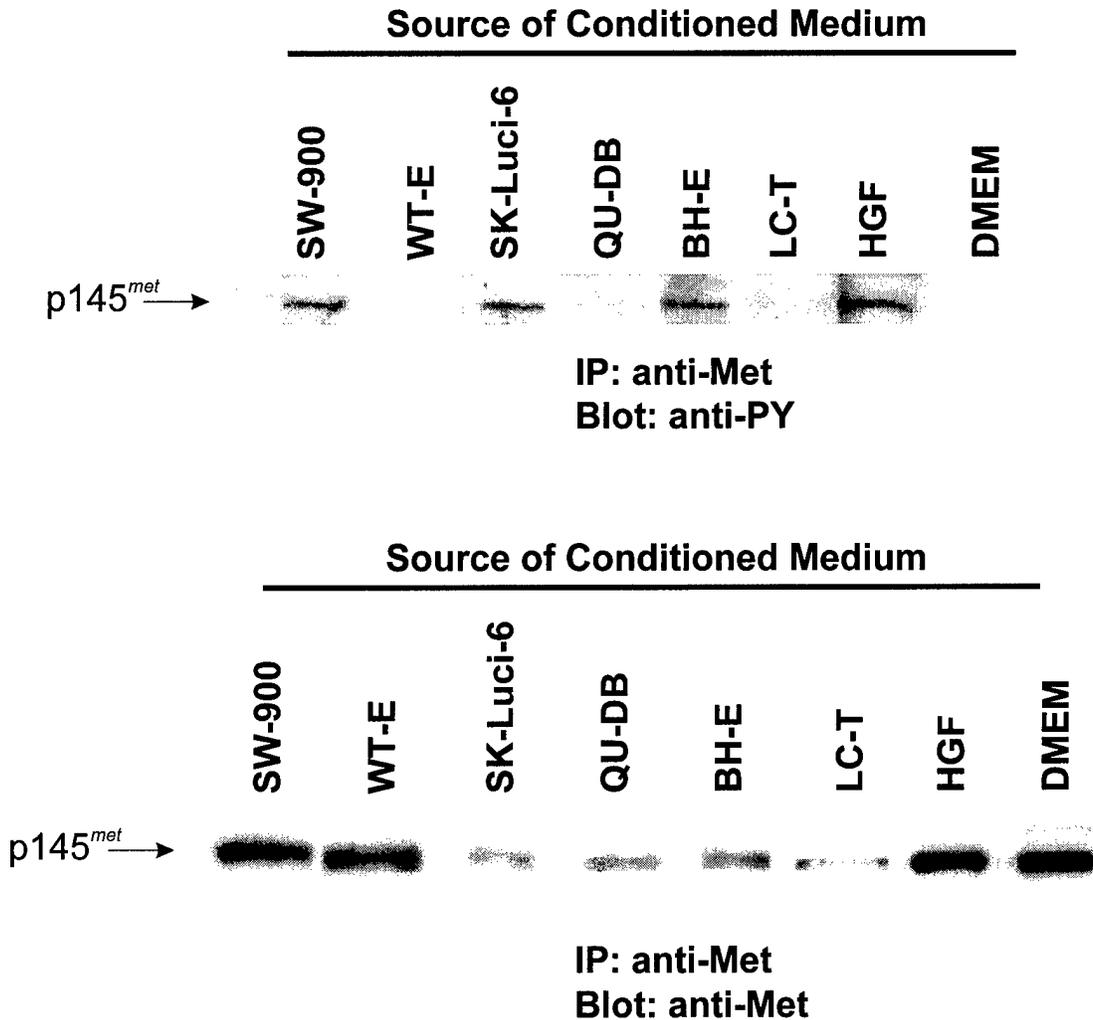
Source of Conditioned Medium

p145^{met} →

IP: anti-Met
Blot: anti-PY

Source of Conditioned Medium


p145^{met} →

IP: anti-Met
Blot: anti-Met

HGF activity in conditioned media from NSCLC cell lines

A549 cells which express Met, but not HGF, were prestarved before incubation with conditioned media from various cell lines. Controls consisted of cells incubated without, or with, HGF (40 ng/ml). After 30 min of incubation at 37°C, cells were washed with ice-cold PBS, lysed in lysis buffer, and immunoprecipitated with rabbit anti-Met antibody. Immunoprecipitates were washed several times with lysis buffer before being analysed by reducing SDS-PAGE. Proteins were analyzed using western blotting with anti-phosphotyrosine antibody (anti-PY) (upper) or with anti-Met antibody (lower).

Figure 4

A

	A549	SW-900	WT-E	
HGF	+ −	+ −	+ −	

p145^*Met* →   IP: anti-Met
Blot: anti-PY

p145^*Met* →   IP: anti-Met
Blot: anti-Met

B

	A549	SW-900	WT-E
HGF	+ −	+ −	+ −

p145^*Met* →

In vitro autophosphorylation
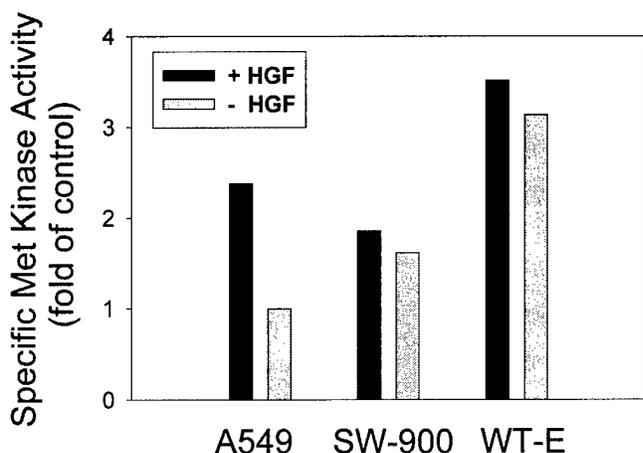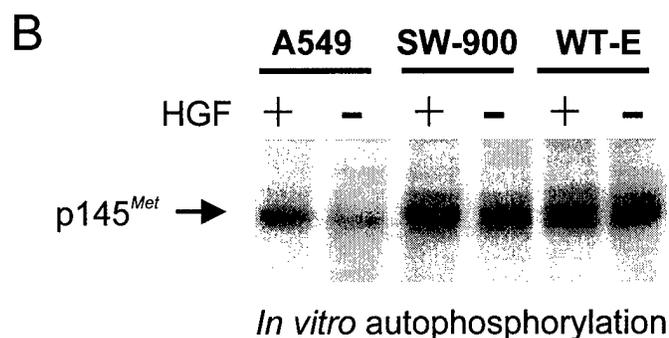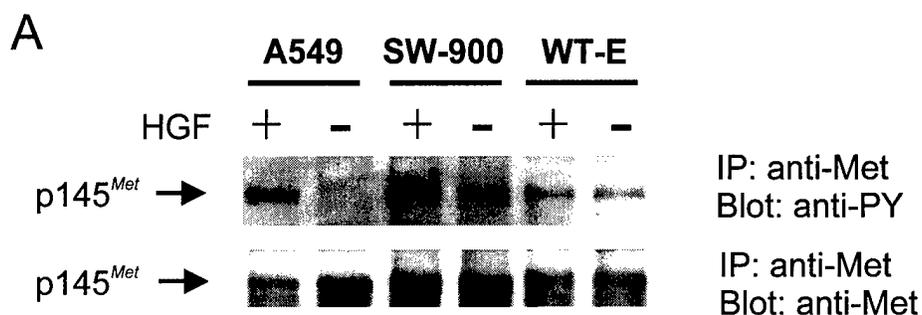
Met is constitutively active in SW-900 and WT-E cells

A549, SW-900 and WT-E NSCLC cell lines were cultured to 80% confluence and serum-starved overnight. The cells were then treated with HGF (20 ng/ml) for 20 min at 37°C and lysed. Clarified cell extracts were normalized for protein concentration and precipitated with anti-Met IgG. Panel A: Half of the immunoprecipitates were analysed by western blotting. The blot was probed with anti-PY antibody, and the bands were visualized with ECL reagents. The same blot was stripped and re-probed with anti-Met IgG as a loading control. Panel B: Half of the immunoprecipates were assayed for Met kinase activity *in vitro* (upper) (See App. II). The signal densities were measured with a PhosphoImager and plotted relative to the control A549 cells without HGF (lower).

Figure 5

A

B
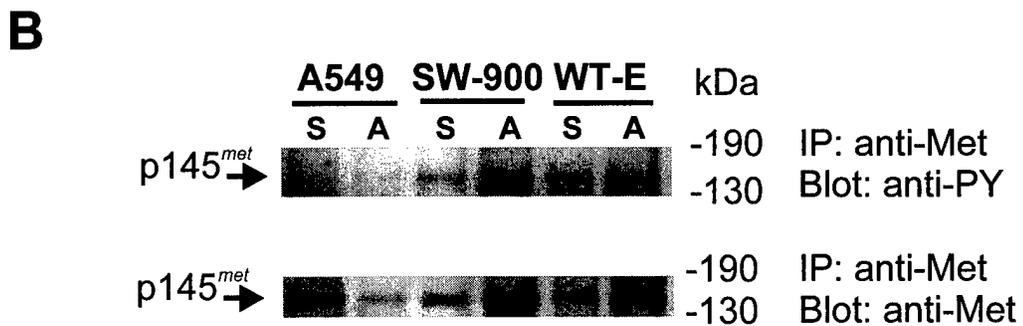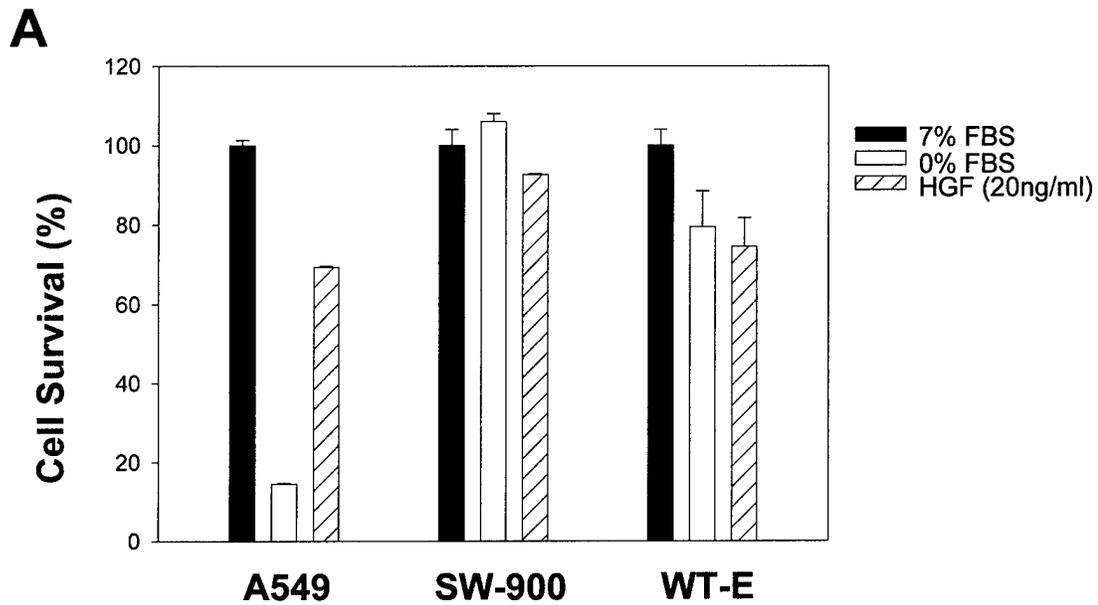
SW-900 and WT-E cells show a high level of survival under anchorage-independent conditions

Panel A: A549, SW-900 and WT-E cells were serum-starved overnight, and seeded in suspension cultures with the treatments as indicated. After 24 h incubation at 37°C, cells were transferred to a 96 well plate and surviving cells were measured with an MTS colorimetric assay. The results are expressed as mean ± range of duplicates, and are representative of two experiments. Panel B: The cells were serum-starved and put in suspension (S) or left on plates (A) for 4 h in 37°C. The cells were then lysed and cell extracts were immunoprecipitated with anti-Met IgG and analyzed by western blotting with anti-PY or anti-Met antibodies.

Figure 5

C

A549 — ^{3}H-Thymidine Incorporation (% of control) plotted against HGF Concentration (ng/ml): 0, 25, 50, 100

SW-900 — values up to 500

WT-E

LC-T

HGF Concentration (ng/ml)

D

	A549	SW-900	WT-E
HGF	+ −	+ −	+ −

p44
p42

Blot: anti-p-ERK1/2

p42 →

Blot: anti-ERK2
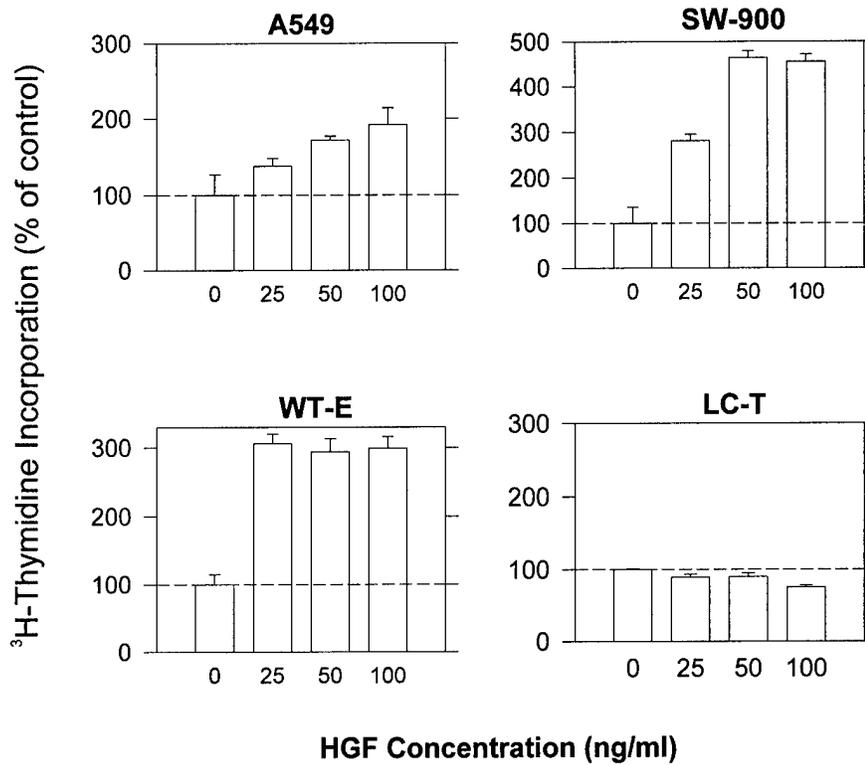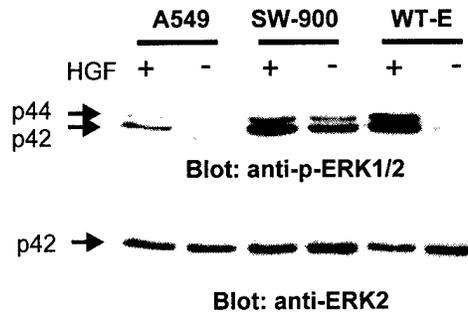
Exogenous HGF stimulates DNA synthesis and phosphorylation of ERK 1/2 in A549, SW-900 and WT-E cell lines

Panel C: Cells were serum-starved overnight and then treated without, or with, HGF at the concentrations indicated. A control consisted of the LC-T cell line (HGF and Met negative). After 24 h, 0.2 μCi of [^{3}H]thymidine was added, and cells were incubated for an additional 24 h. Cells were then harvested, transferred to filters, and the incorporation of [^{3}H]thymidine was measured using a scintillation counter. Results are expressed as the mean cpm of quadruplicate wells ± S.D. Panel D: The cells were serum-starved overnight and treated without, or with, HGF (20 ng/ml) for 20 min and then lysed. The cell extracts were analyzed by western blotting with anti-phospho-ERK1/2 antibody. The same blot was stripped and re-probed with anti-ERK2 antibody to confirm equal protein loading between groups.

Figure 6

A

B
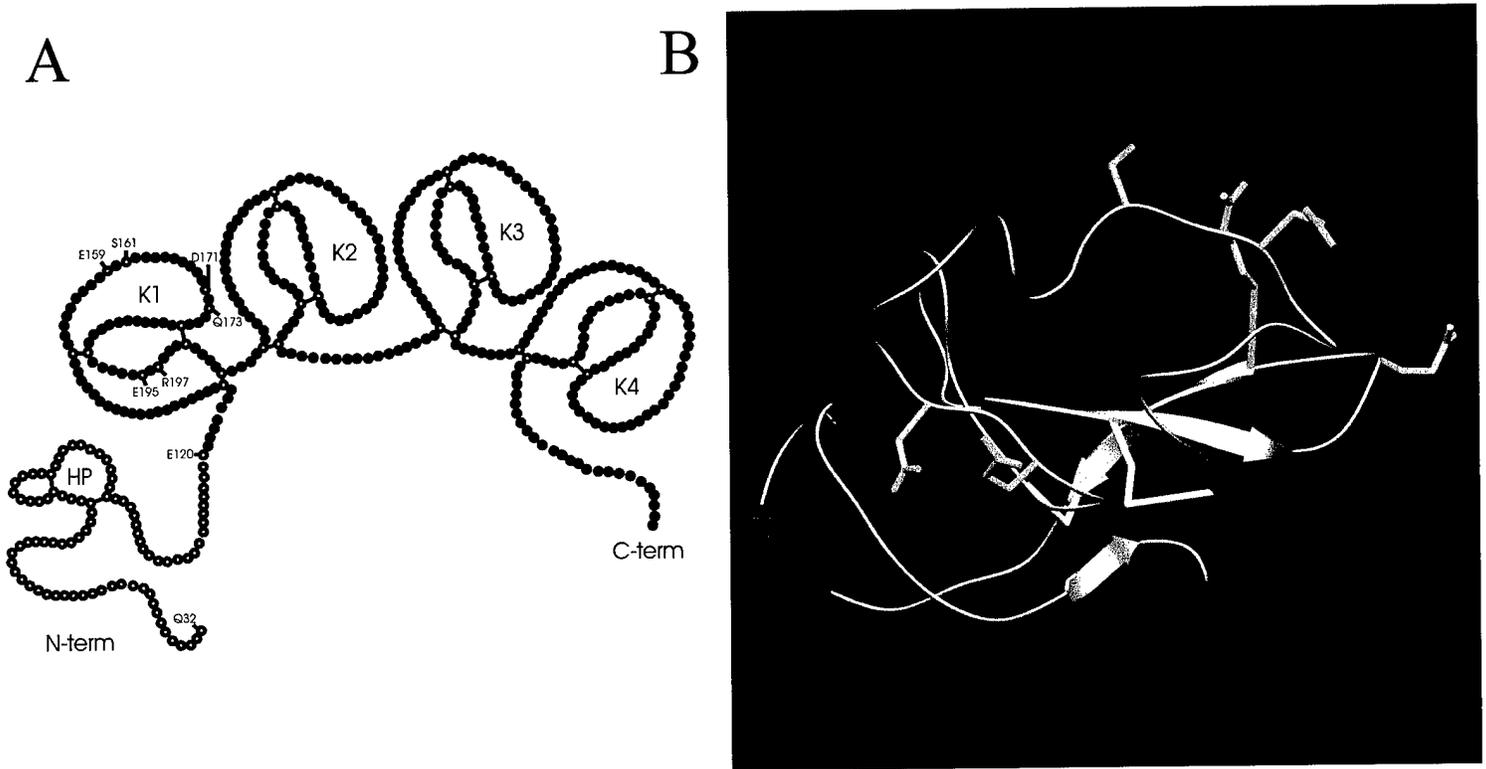
Structure of HGF

Panel A: α-chain of HGF. The hairpin loop (HP), the four kringle domains (K1, K2, K3, K4) are indicated in the diagram. Two mutation-sensitive patches were identified in the K1 domain. The first patch consists of amino acids E159, S161, E195, R197, all of which are predicted to be close to each other in the tertiary structure of K1. The second patch, on the oposite side of K1, consists of D171 and Q173. A peptide in the N-terminal domain (Q32-E120) was used for immunization of rabbits.

Panel B: Modelled K1 domain of the alpha chain of HGF. The backbone is illustrated in ribbon representation and the side chains implicated in Met binding are shown. (Modelled by Dr. Z. Jia, Dept. of Biochem.)

Appendix I

IDENTIFICATION OF PARACRINE AND AUTOCRINE HEPATOCYTE GROWTH FACTOR LOOPS IN NON-SMALL CELL LUNG CARCINOMAS

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Running title: HGF paracrine and possible autocrine loop in lung carcinomas

Keywords: HGF, Met, protein secretion, cell proliferation, Metastasis, lung cancer

Manuscript in preparation

ABSTRACT

Hepatocyte growth factor (HGF) is secreted from mesenchymal cells and stimulates multiple functions including mitogenesis, motogenesis and morphogenesis of epithelial cells in many tissue types. HGF shows increased expression in human non-small cell lung carcinomas (NSCLC), but its role in tumor progression is not clearly known. We have examined expression of HGF and its receptor, Met, in NSCLC cell lines derived from primary tumor and pleural effusions of lung cancer patients. Using semi-quantitative RT-PCR and western blotting, we showed that 6/6 NSCLC cell lines expressed Met mRNA, but only two cell lines expressed detectable Met protein, implying incomplete translation or processing of Met in some carcinomas. Similarly, all NSCLC cell lines expressed significant or trace levels of HGF mRNA as determined using RT-PCR with direct radiolabelling of products, and 4/6 cell lines expressed detectable HGF protein. In contrast, 12 small cell lung carcinoma cell lines examined showed no detetable HGF or Met protein. Two HGF-expressing cell lines showed constitutive tyrosine-phosphorylation of Met, consistent with establishment of a functional autocrine HGF loop. Under serum-free detached conditions, these cells showed a high level of cell survival, compared to an HGF-negative, Met-positive cell line. These findings indicate that activation of an HGF autocrine loop occurs in some NSCLC cell lines and is associated with sustained survival of detached carcinoma cells; although additional paracrine stimulation by HGF is required for a mitogenic response. Thus autocrine HGF loops may provide a survival stimulus during early stages of tumor progression in NSCLC.

INTRODUCTION

Growth factors which act in a paracrine or autocrine manner are important regulators of stromal-tumor interactions in both normal and malignant cell development. Hepatocyte growth factor (HGF) is secreted primarily from mesenchymal/stromal cells of many different tissues (1-3); whereas HGF receptor (Met) expression is detected in a variety of epithelial (and some non-epithelial) cells in a broad range of tissues (4). HGF is secreted as an inactive single-chain pro-HGF protein of 105 kDa (5). Pro-HGF is cleaved by serine proteinases at Arg494-Val495, and is converted to a heterodimeric mature HGF molecule consisting of disulfide-linked α (65 kDa) and β (30 kDa) chains of 463 and 234 amino acid residues, respectively (6). Processing of HGF is required for its biological activities. Several known serine proteinases including urokinase (7) and tissue-type plasminogen activator (8), a protease homologous to blood coagulation factor XII (9), HGF converting enzyme (10), and other related HGF activating proteases (11), have been shown to activate HGF. Its activity can also be affected by association with extracellular matrix proteins such as proteoglycans, e. g. heparin (12). The *met* proto-oncogene product, a member of the tyrosine kinase receptor family, has been identified as the HGF receptor (13). Ligand binding induces kinase activation and tyrosine phosphorylation of Met (14). A two tyrosine motif in the COOH terminal tail of Met acts as a multifunctional docking site for SH2 domain-containing transducer proteins, resulting in stimulation of HGF-induced functions (15; 16). Activation of Met by HGF affects a variety of epithelial cell functions including cell proliferation (17; 18), survival (19), differentiation (20), cell motility (21), invasion (21), and angiogenesis (22).

Met is frequently over-expressed or amplified in many types of human cancers including non-small cell lung carcinomas (NSCLC) (23), breast (24), ovarian (25), and colorectal carcinomas

(26), melanomas (27), and osteosarcomas (28). We (24) and others (29) have previously shown strong expression of HGF mRNA in regions of invasive human breast cancer compared to more heterogeneous, weak, expression in ductal carcinoma *in situ* and nonmalignant epithelium. HGF is also over-expressed and often activated in NSCLC tissues (30; 31), and some small cell lung carcinoma (SCLC) cell lines (32; 33). This high level of HGF expression correlates with poor survival of cancer patients (29; 34). In contrast, HGF expression in corresponding normal lung epithelium is low (31; 35; 36), although expression of HGF by some bronchial epithelial and carcinoma cells *in vitro* has been shown (33; 37). Together, these findings suggest that establishment of an HGF autocrine loop may provide a selective advantage for autonomous growth and metastasis of carcinoma cells. This view was further promoted by the demonstration that expression of HGF (38) or a constitutively active mutant form of Met (Tpr-Met) (39) in transgenic mice, or in transformed cell lines (40; 41), promotes formation of tumors and metastasis. Altered expression of functionally-active HGF in epithelial cells could occur at several levels including transcription, and post-translational modification by enzymatic processing.

As a first step to examine the role of paracrine and autocrine HGF loops in NSCLC, we have used semi-quantitative RT-PCR and western blotting to examine the expression of HGF and Met mRNA and protein in established NSCLC cell lines. HGF protein was purified from conditioned medium, using copper(II) affinity chromatography, based on the ability of HGF to bind to Cu(II) (42). The activity of HGF protein secreted from different carcinoma cell lines was assessed by its ability to induce tyrosine- phosphorylation of Met in the lung carcinoma cell line, A549. The level of tyrosine-phosphorylation of Met, cell survival, and DNA synthesis was compared under

conditions of paracrine and autocrine stimulation by HGF. Our results suggest differential effects of paracrine and autocrine stimulation by HGF in NSCLC cells.

MATERIAL AND METHODS

Antibodies. Mouse anti-phosphotyrosine (PY20) monoclonal antibody was purchased from BD Transduction Laboratories (San Jose, CA). Mouse anti-human Met IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sheep anti-human HGF IgG were received from Genentech Inc. (San Francisco, CA).

Human lung carcinoma cell lines. Human NSCLC cell lines SW-900 (43), WT-E (45), SK-Luci-6 (46), QU-DB (47), BH-E (45), LC-T (48) were established from primary tumor or pleural fluids from lung cancer patients. In some experiments a nonmalignant human bronchial epithelial cell line (HBE) was also used (49). Cell lines were routinely grown in RPMI 1640 medium, supplemented with 7% fetal bovine serum, and were confirmed to be mycoplasma negative as previously described (50).

PCR primers. The primers were designed according to the sequences of HGF and Met from Genbank using PC/GENE computer software. Primers were engineered to detect HGF and Met cDNA in both human and mouse, but not the homologous family members MSP (51) and Ron (52), respectively. The primers for HGF amplification were: sense primer 5' - TGT CGC CAT CCC CTA TGC AG - 3' located from position 69 to 88, and antisense primer 5' -TCA ACT TCT GAA CAC TGA GG - 3' located from position 610 to 629. The PCR product for HGF PCR amplification is 560

bp long (Figure1). The primers for Met amplification were: sense primer 5' - CCA CTA CAA CAT GAG CAG CC - 3' located from position 351 to 371, and antisense primer 5' - CTC CCT GCA GGT TTT GAT GC - 3' located from position 541 to 560. The PCR product for Met PCR amplification is 206 bp long (data not shown). In addition, primers for two house-keeping genes were used: glucuronidase B (GUSB) (53) and transferrin receptor (obtained from Dr. J. Gerlach).

RT-PCR analysis. Total RNA from various NSCLC tissues and cell lines was extracted using TriZol reagent (Canadian Life Technologies, Burlington, ON)), and used in a reverse transcriptase reaction to produce cDNA using a First-Strand Synthesis Kit (Amashan-Pharmacia Biotech, Baie d'Urfe, Que). PCR reactions were carried out without, or with, direct labelling of primers with $[\alpha-^{32}P]dATP$ (Mandel Scientific, Guelph, ON). cDNA (50 ng) was added to each 10 µl reaction, which also contained 20 mM Tris (pH 8.3), 50 mM KCl, 0.2 mM dNTPs, 1.5 mM $MgCl_2$. Oligonucleotide primers specific for HGF and GUS B (internal control), or Met and transferrin receptor (internal control) were added as indicated. GUS B and transferrin were chosen as controls since their mRNA levels were found empirically to correspond to those of HGF and Met, respectively. The reaction was initiated by the addition of 1 U of Taq polymerase (Canadian Life Technologies), and samples were then incubated at 95°C for 1 minute (denaturing), 55°C for 1 minute (annealing), 72°C for 1 minute (elongation). Unlabelled and labelled PCR was carried out with 25 cycles. The reaction products were resolved on a 1.5% agarose gel. Unlabelled PCR products were stained with ethidium bromide and visualized under UV light illumination. Labelled PCR products were measured in gel slices using a liquid scintillation counter.

Copper (II) affinity chromatography. Purification of HGF was carried out using Cu (II) affinity chromatography based on the Cu(II) binding ability of HGF (42). The principal of separation of HGF from biological samples by Cu(II) affinity chromatography is based on the fact that HGF has several cationic sequences (His-X-His) in the kringle domains of the HGF molecule. Five ml of each conditioned medium were loaded onto a 1 ml Cu(II) chelating column, which had been equilibrated with equilibration buffer (20 mM sodium phosphate, pH 7.2, 1 M NaCl, 1 mM imidazole). HGF protein bound specifically to the copper (II) affinity column, and unbound proteins were washed away with 15 column volumes of the same buffer. HGF protein was eluted using equilibration buffer containing 80 mM imidazole, and the eluant was concentrated using an Amicon Microcon-10 concentrator before being analyzed on 8% SDS PAGE under reducing conditions. Proteins were then transferred onto nitrocellulose membrane and probed with a sheep anti-human HGF antibody (Genentech Inc.). The bands were revealed using an ECL kit (Amersham Pharmacia Biotech).

Immunoprecipitation and western blot analysis. NSCLC cell lines were grown to 80% confluence and serum-starved for 24 hour. Cell lines were then rinsed with phosphate-buffered saline and lysed in a lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA, 1% Nonidet P-40, 1 mM Na_3VO_4, 50 mM NaF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged for 10 minutes at 14,000 rpm in an IEC/Micromax centrifuge at 4°C. Protein concentration of supernatants was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Equal amounts of protein from cell lysates were immunoprecipitated with the indicated antibodies at 4°C for 2 h. Immunoprecipitates were collected on protein A-Sepharose (Amersham Pharmacia Biotech), washed three times with lysis buffer, and subjected to 8% SDS-

PAGE under reducing conditions, followed by western blotting with the indicated antibodies. Immunoreactive bands were revealed using an ECL kit.

Met kinase assay. Cells were cultured to 80% confluence and serum starverd overnight. The cells were then treated without or with HGF (20ng/ml) for 20 min at 37 °C. The cell were lysed and extracts were normalized for protein concentration and precipitated with anti-Met IgG. The immunoprecipitates were washed twice with lysis buffer and once with kinase buffer containing 20 mM Pipes (pH 7.0), 10 mM $MnCl_2$ and 10 μM Na_3VO_4 and resuspended in cold kinase buffer. The reaction was initiated by addition of 10μCi [γ-^{32}P]ATP. The reaction was carried out at 30 °C for 10 min and stopped by adding sample buffer and boiling for 3 min. The samples were subjected to 8 % SDS-PAGE and the gel was treated with 1 M KOH for 30 min at 45 °C to hydrolyse Ser/Thr phosphorylation sites. The gel was then fixed in 45% MeOH and 10% acetic acid for 30 min at room temperature and dried. The autophosphorylation of Met was analyzed by a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Met activation Assay for HGF activity. The human lung carcinoma cell line, A549, which expresses Met, does not produce HGF protein in our system, was used as a read-out for Met activation. A549 cells were exposed for 30 min to conditioned media from various NSCLC cell lines, HGF (20 ng/ml), or medium alone. The cells were lysed and subjected to immunoprecipitation with anti-Met antibody. Immunoprecipitates were subjected to 8% SDS-PAGE under reducing conditions, and immunoblotting was carried out with anti-phosphotyrosine antibody or anti-Met antibody (loading control). Immunoreactive bands were revealed using an ECL kit.

Cell survival. Carcinoma cells were cultured in tissue culture plates and pre-starved overnight, harvested and seeded at a density of 2×10^4 cells in 1.5 ml of RPMI 1640 medium containing 0.5 mg/ml BSA in 0.6% agar-coated 35 mm Corning non-tissue culture plates. After 24 h incubation at 37°C with indicated treatment, cells were collected and centrifuged in Eppendorf tubes (1000 rpm for 5 min), replated in a 96-well plate with 7% FBS/RPMI medium, and incubated at 37°C for 4 h. Cell survival was then measured using a colorimetric method based on the conversion of MTS tetrazolium to formazan (CellTiter aqueous Kit, Promega, Madison, WI).

DNA synthesis. Carcinoma cells (1×10^4) were incubated in triplicate in a 24 well plate for 24 hours at 37°C and 5% CO_2, alone, or with HGF (20 ng/ml). After 24 hours, 0.2 µCi of [^{3}H]thymidine (Amersham Pharmacia Biotech) was added, and cells were incubated for an additional 24 hours period. Cells were harvested and aliquots of cells (1000 per well) were placed in 96-well microtiter plates and transferred to filters using a Titertek cell harvester (ICN, Costa Mesa, CA), and DNA synthesis was measured as incorporation of [^{3}H]thymidine in a scintillation counter (Beckman, Mississauga, ON, Canada).

RESULTS

Expression of HGF and Met mRNA in non-small cell lung carcinoma tissues and cell lines: The level of HGF and Met mRNA was first examined using RT-PCR analysis. The linearity of the PCR reaction was assessed to establish the optimal reaction conditions, and the reaction was performed

with internal controls using β glucuronidase (GUS B) for HGF, and transferrin receptor for Met. The PCR reaction was carried out with 25 cycles which was found to be linear. Human NSCLC cell lines SK-Luci-6, SW-900 and BH-E expressed significant levels of HGF mRNA detected using both unlabelled and labelled PCR; whereas WT-E, LC-T and QU-DB cell lines showed trace levels of HGF mRNA detected only using radiolabelled PCR (Figure 1 and Table I). A nonmalignant human bronchial epithelial cell line, HBE, also showed trace amounts of HGF mRNA expression. All cell lines tested showed detectable levels of Met mRNA expression (Table I).

Expression of HGF and Met protein in non-small cell lung carcinoma cell lines: To detect HGF protein secreted by carcinoma cell lines, we employed Cu (II) affinity chromatography (42) to isolate HGF from conditioned media. Western blotting of purified protein was carried out and putative HGF protein was detected with sheep anti-human HGF antisera which recognizes both α and β chains of the HGF molecule. Conditioned media from NSCLC cell lines SW-900, WT-E, SK-Luci-6 and BH-E all showed detectable expression of HGF protein. However, conditioned media from NSCLC cell lines QU-DB, and LC-T showed no detectable HGF protein (Figure 2). Met protein was detected in cell lysates of two HGF-secreting NSCLC cell lines (WT-E and SW-900); but not in the remaining cell lines (Table I). In contrast, HGF and Met protein were not expressed in a series of 12 small cell lung carcinomas tested (Table II).

HGF activity from conditioned media (CM) of non-small cell lung carcinoma cells: To assess the activity of HGF in CM from carcinoma cell lines, we tested the ability of CMs to activate Met in A549 carcinoma cells (which are Met positive, HGF negative). A control showed strong tyrosine-

phosphorylation of Met in A549 cells incubated with exogenous HGF (40 µg/ml), compared to cells incubated in media alone. Three out of four HGF-containing conditioned media from human NSCLC cell lines tested stimulated tyrosine phosphorylation of Met in A549 cells (Figure 3). These results indicate that the majority of HGF-producing NSCLC cell lines tested secrete active HGF protein.

Status of tyrosine-phosphorylation of Met in non-small cell lung carcinoma cell lines: A prediction from these studies is that carcinoma cell lines that express both active HGF and Met would show constitutive activation of Met, consistent with the establishment of an HGF autocrine loop in these cells. To test this possibility, we examined the tyrosine-phosphorylation level of Met in two NSCLC cell lines (WT-E and SW-900) without, or with, incubation with HGF (20 ng/ml), using western blot analysis. Both cell lines which co-express HGF and Met, showed significant tyrosine-phosphorylation of Met even without treatment with exogenous HGF. These two cell lines were also assayed for Met kinase activity *in vitro* and they showed consititutively active Met without HGF stimulation (Figure 4).

Cell survival under anchorage-independent conditions: To test the biological function of putative paracrine versus autocrine activation of Met in NSCLC cell lines, we examined cell survival in A549, SW-900 and WT-E cell lines. SW-900 and WT-E cells, with autocrine expression of HGF and sustained tyrosine-phosphorylation of Met, consistently showed high level of cell survival under nonadherent serum-starved conditions; whereas A549 cells which express Met but not HGF showed a reduced survival response (Figure 5A). In addition, SW-900 and WT-E cells, not A549 cells,

showed a sustained high level of Met phosphorylation when cells were kept in suspension, which correlated with the cell survival under anchorage-independent conditions (Figure 5B).

DNA synthesis and phosphorylation state of ERK in non-small cell lung carcinoma cell lines: We also examined DNA synthesis in A549, SW-900 and WT-E cell lines. Interestingly, the base level of DNA synthesis in the absence of exogenous HGF was low in all cell lines, regardless of the level or activity of secreted HGF or Met kinase activity, and they showed strong paracrine stimulation by HGF of DNA synthesis. As a control, LC-T (HGF and Met negative) showed no stimulation. (Figure 6 and Table I). The phosphorylation of ERK1/2 was increased by addition of exogenous HGF to the cells (Figure 7). Altogether, these results suggest that increased cell survival, but not DNA synthesis, correlated with the expression of HGF and autophosphorylation of Met at tyrosine residues in NSCLC cell lines.

DISCUSSION

A broad range of genetic markers have ben associated with invasive lung cancer including amplification of EGF receptor (55), K-ras point mutations (56), and dominant negative mutations of p53 (57). However, to date there are no reliable genetic markers for premalignant changes in lung epithelia. Previous studies have shown that HGF is over-expressed and often activated in the majority of NSCLC tissues compared to adjacent normal lung tissue (30,31), and this high level of HGF expression has been identified as a possible independent predictor of poor survival of lung cancer patients (30). Met is also frequently over-expressed in NSCLC, particularly in regions of

invasive adenocarcinomas and large cell undifferentiated carcinomas (23). Earlier studies with NSCLC cell lines have demonstrated autocrine HGF expression and function in a lung bronchial epithelial and carcinoma cell line (33), and autocrine stimulation of motility by a two-kringle variant of HGF in a small cell lung carcinoma cell line (37). More recent results from Yi *et al.* (32) have shown expression of HGF mRNA in a broad range of NSCLC cell lines, however the expression of active HGF protein and existence of possible paracrine and autocrine HGF loops was not fully investigated in the study.

To assess the presence of possible paracrine and autocrine HGF loops, we first examined HGF and Met expression and function in six NSCLC cell lines established from primary tumors and pleural effusions (See Table I). Our study involved a combined analysis of HGF and Met mRNA and protein expression, and functional roles of paracrine and autocrine activation of Met. The results showed that all of the NSCLC cell lines tested expressed detectable HGF mRNA, of which four cell lines secreted HGF protein. Western blot analysis of proteins resolved by SDS-PAGE under reducing conditions showed bands corresponding to both pro-HGF (100 kDa) and mature alpha chain (65 kDa). Similarly, Met mRNA was detected in all NSCLCs, however only two cell lines (SW-900 and WT-E) expressed significant levels of Met protein. The differences in Met and HGF mRNA versus protein detected could represent different sensitivities of the assays, or differences in post-transcriptional or postranslational steps in the expression of HGF and Met in NSCLC cells. Conditioned media from only three of the HGF-producing cell lines (SW-900, SK-Luci-6, and BH-E) showed biologically active as determined by the ability to stimulate tyrosine-phosphorylation of Met in A549 cells (Met postive, HGF negative). The lack of detectable HGF activity in HGF-containing CM from WT-E cells could reflect sensitivity of the assay, or inactivation of HGF due to association with extra cellular matrix proteins (12) or degradation (58).

Although expression of secreted HGF and Met was detected in NSCLC cell lines derived from adenocarcinomas, squamous cell carcinomas, and large cell anaplastic carcinomas, the number of cell lines examined was insufficient for statistical correlation to specific NSCLC histotypes. In addition, some NSCLC cell lines expressed HGF, but not Met, and some expressed neither HGF nor Met. In contrast, no expression of HGF or Met was detected in 12 small cell lung carcinoma cell lines examined (data not shown). Together these findings imply that both paracrine and autocrine HGF loops may be important in development of NSCLC.

Two NSCLC cell lines (SW-900 and WT-E) which express high levels of Met, also secreted HGF protein, demonstrating the existence of a putative autocrine HGF loop in a significant proportion of NSCLC cells. Our findings further showed tyrosine-phosphorylation of Met and increased Met kinase activity in both cell lines, suggesting the presence of an autocrine HGF loop in these cells. Interestingly, conditioned media from WT-E cells showed no detectable HGF activity, although HGF protein was evident (Figure 2). Thus, WT-E cells , which showed a high level of activated Met, may exhibit intracrine activation of Met via association with the cytoplasmic pool of HGF inside the cell, as has been shown for fibroblast growth factor receptor (60) and epidermal growth factor receptor (61). Alternatively, activity of soluble HGF may be blocked by association with proteoglycans such as heparin sulphate (18).

To further assess the functional role of paracrine and autocrine activation of Met in NSCLC cells, we determined the cell survival and DNA synthesis responses without, or with, addition of exogenous HGF. Interestingly, all Met-expressing NSCLC cell lines required paracrine stimulation with HGF for an optimal proliferation response, regardless of the presence of an autocrine HGF loop. Two NSCLC cell lines (SW-900 and WT-E) which express an autocrine HGF loop showed a sustained high level of Met tyrosine-phosphorylation and cell survival under nonadherent conditions.

In an independent study, Yi *et al*, (32) showed that NSCLC cell lines expressing HGF mRNA exhibited paracrine, but not autocrine, stimulation by HGF of DNA synthesis; however the level of constitutive activation of Met was not assessed in their study. Our results indicate that autocrine activation of Met is sufficient to sustain survival of NSCLC cells, whereas additional paracrine stimulation with HGF is required to stimulate DNA synthesis.

In summary, our panel of NSCLC cell lines represent different stages of tumor progression including acquisition of an autocrine HGF loop, and concomitant loss of anchorage requirement for cell survival and growth. Our findings further raise the possibility that paracrine and autocrine stimulation of Met triggers different functional responses in NSCLC cells, possibly due to quantitatively or qualitatively different signalling patterns. Experiments are in progress to further assess the pattern of HGF-induced signalling and cell functions associated with paracrine versus autocrine HGF stimulation of NSCLC cells.

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TABLE I

Expression of HGF and Met in human Non-small cell lung carcinoma cell lines

Carcinoma Cell Lines	Histology	Origin	HGF			Met		HGF-induced DNA synthesis[c]
			mRNA	Protein	Activity	mRNA	Protein	
SW-900	squamous cell carcinoma	primary tumor	+[b]	+	+	+	+	+
WT-E	squamous cell carcinoma	pleural effusion	+/-	+	-	+	+	+
SK-Luci-6	large cell anaplastic	primary tumor	+	+	+	+	-	-
QU-DB	large cell anaplastic	primary tumor	+/-	-	-	+	-	-
BH-E	adenocarcinoma	pleural effusion	+	+	+	+	-	-
LC-T	adenocarcinoma	primary tumor	-	-	-	+	-	-

Legend:

a) See Materials and Methods for designation of Non-small cell lung carcinoma cell lines.

b) N/D, not determined; +, positive; +/-, trace amount; -, negative.

c) Cells (1×10^4) in triplicate were incubated in a 24 well plate for 24 hours at 37°C and 5% CO_2, alone, or with HGF (20 ng/ml). After 24 h, ^{3}H-Thymidine was added, and cells were incubated for a second 24 h period. DNA synthesis was measured as incorporation of ^{3}H-Thymidine.

TABLE 2

Expression of HGF and Met in human small cell lung carcinoma cell lines

Carcinoma Cell Lines[a]	Origin	HGF			Met	
		mRNA	Protein	Activity	mRNA	Protein
BK-T	primary tumor	-[b]	-	-	+	-
CK-A	needle aspiration	-	-	-	+	-
H69	pleural effusion	-	-	-	+	-
H128	pleural effusion	-	-	-	+	-
HA-E	pleural effusion	-	-	-	+	-
HG-E	pleural effusion	-	-	-	+	-
LG-T	lymph node biopsy	-	-	-	+	-
MM-1	pleural effusion	-	-	-	+	-
SHP-77	pleural effusion	-	-	-	+	-
SM-E	pleural effusion	-	-	-	+	-
SV-E	pleural effusion	-	-	-	+	-
YR-A	needle aspiration	-	-	-	+	-

Legend:

a) See Materials and Methods for designation of non-small cell lung carcinoma cell lines, and details of procedures for mRNA and protein analysis of HGF and Met.

b) N/D, not determined; +, positive; +/-, trace amount; -, negative.

FIGURE LEGENDS

Figure 1. Analysis of NSCLC cell lines: Total RNA from various NSCLC cell lines was extracted

and used in a reverse transcription reaction to produce cDNA. A nonmalignant lung bronchial cell line,

HBE (59), was used as a control. Primers specific for HGF and GUS B (as internal control) were added

to the cDNA. Unlabelled (Panel A) and labelled (Panel B) PCR was carried out with 25 cycles, and the

reaction products were analysed as described in Materials and Methods.

Figure 2. Detection of HGF protein in conditioned media of NSCLC cell lines: The presence of

HGF protein in the conditioned media collected from different lung carcinoma cell lines was

determined using copper (II) affinity chromatography to purify putative HGF from conditioned media.

Fractions containing putative HGF were concentrated by Microcon concentrators, and were analysed

by reducing SDS-PAGE, followed by western blotting with sheep anti-HGF antibody (Genentech).

Immunoreactive bands were revealed by ECL. Arrows corresponding to pro-HGF and mature HGF

are shown.

Figure 3. HGF activity in conditioned media from NSCLC cell lines: A549 cells which express

Met but not HGF were prestarved before incubation with conditioned media from various cell lines.

Controls consisted of cells incubated without, or with, HGF (40 ng/ml). After 30 min of incubation at

37°C, cells were washed with ice-cold PBS, lysed in lysis buffer, immunoprecipitated with rabbit anti-

Met antibody. Immunoprecipitates were washed several times with lysis buffer before analysed by

reducing SDS-PAGE. Proteins were analyzed using western blotting with anti-phosphotyrosine

antibody (anti-PY) (upper) or with anti-Met antibody (lower).

Figure 4: Met is constitutively active in SW-900 and WT-E cells. A549, SW-900 and WT-E NSCLS

cell lines were cultured to 80% confluence and serum-starved overnight. The cells were then treated

with HGF (20 ng/ml) for 20 min at 37°C and lysed. Clarified cell extracts were normalized for protein concentration and precipitated with anti-Met IgG. Panel A: Half of the immunoprecipitates were analysed by western blotting. The blot was probed with anti-PY antibody, and the bands were visualized with ECL reagents. The same blot was stripped and re-probed with anti-Met IgG as a loading control. Panel B: Half of the immunoprecipates were assayed for Met kinase activity *in vitro* as described in Materials and Methods. The signal densities were measured with a PhosphoImager and plotted relative to the control A549 cells without HGF (lower panel).

Figure 5: SW-900 and WT-E cells show a high level of survival and constitutive Met phosphorylation under anchorage-independent conditions. Panel A: A549, SW-900 and WT-E cells were serum-starved overnight, and seeded in suspension cultures with the treatments as indicated. After 24 h incubation at 37°C, cells were transferred to 96-well plates and surviving cells were measured with the MTS colorimetric assay as described in Materials and Methods. The results are expressed as mean ± range of duplicates, and are representative of two experiments. Panel B: The cells were serum-starved and put in suspension (S) or left on plates (A) for 4 h in 37°C. The cells were then lysed and cell extracts were immunoprecipitated with anti-Met IgG and analyzed by western blotting with anti-PY or anti-Met antibodies.

Figure 6. Exogenous HGF induces DNA synthesis in A549, SW-900 and WT-E cell lines: Cells were serum-starved overnight and subcultured in 96-well tissue culture plates (1000 cells per well) without, or with, HGF at the concentrations indicated, as described in the Materials and Methods. A control consisted of the LC-T cell line (HGF and Met negative). After 24 h, 0.2 μCi of [^{3}H]thymidine was added, and cells were incubated for an additional 24 h cells. Cells were then harvested, transferred to filters, and the incorporation of [^{3}H]thymidine was measured using a scintillation counter. Results are expressed as the mean cpm of quadruplicate wells ± S.D.

Figure 7: Exogenous HGF stimulates phosphorylation of ERK in A549, SW-900 and WT-E cells.

The cells were cultured to 80% confluency and serum starved overnight. Cells were treated without, or with, HGF (20 ng/ml) for 20 min and then lysed. The cell extracts were analyzed by western blotting with anti-phosphor-ERK1/2 antibody. The same blot was stripped and re-probed with anti-ERK2 antibody to confirm equal protein loading between groups.

Figure 1

Figure 2

Figure 3

Source of Conditioned Medium

p145 ^{met} ⟶

IP: anti-Met
Blot: anti-PY

Source of Conditioned Medium

p145 ^{met} ⟶

IP: anti-Met
Blot: anti-Met

Figure 4

A

B

In vitro autophosphorylation
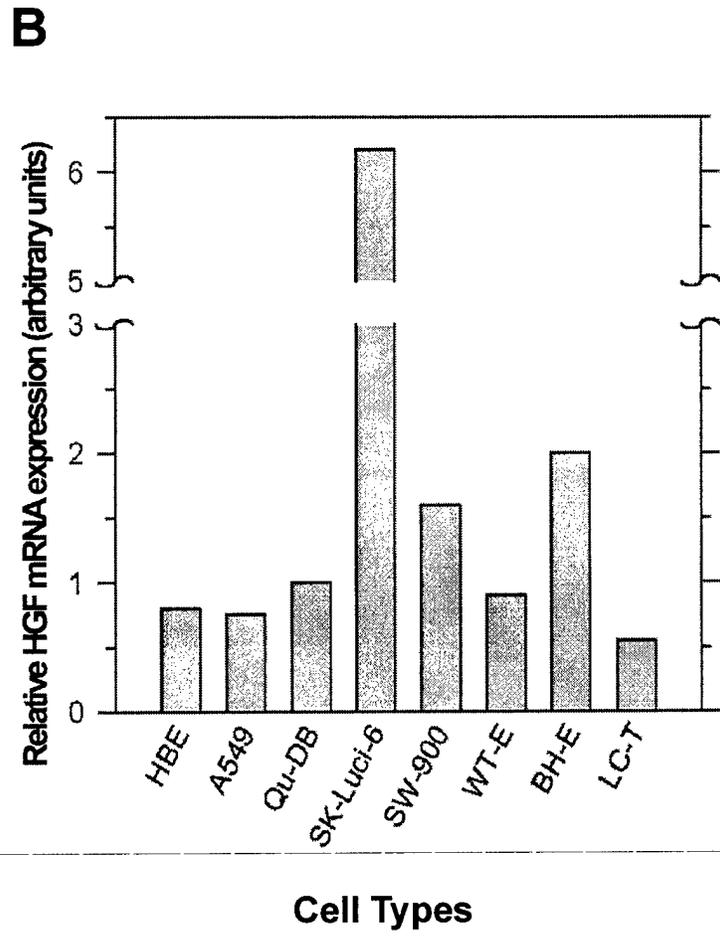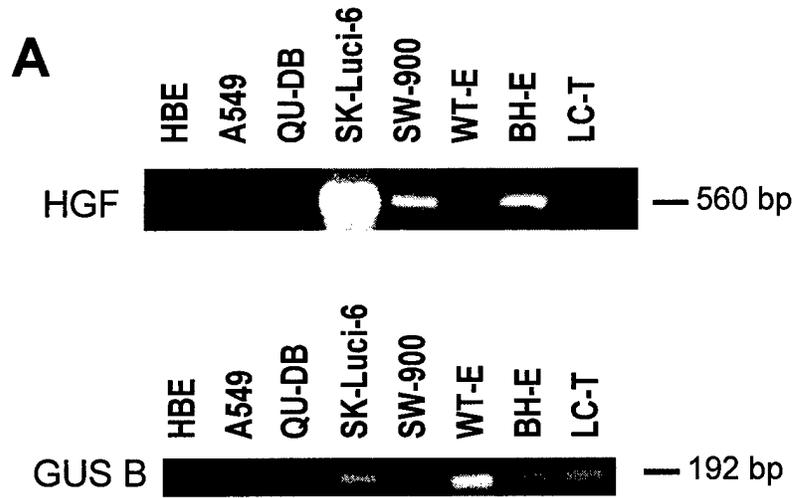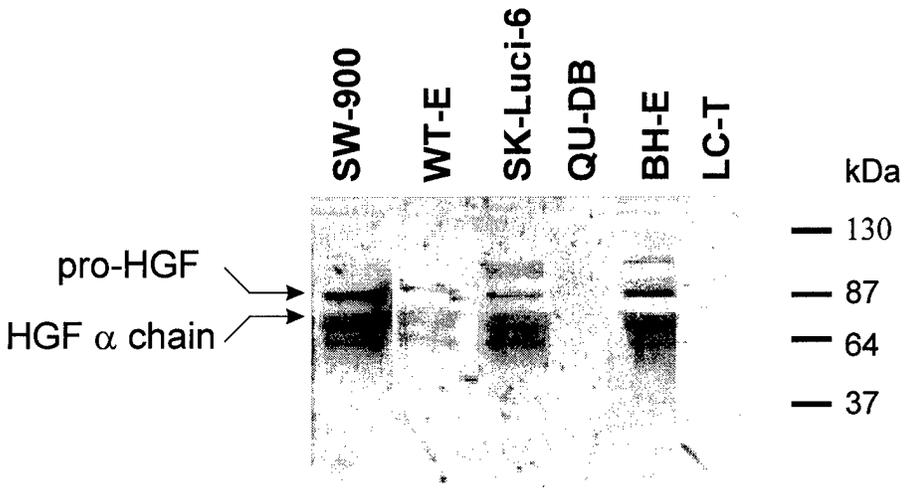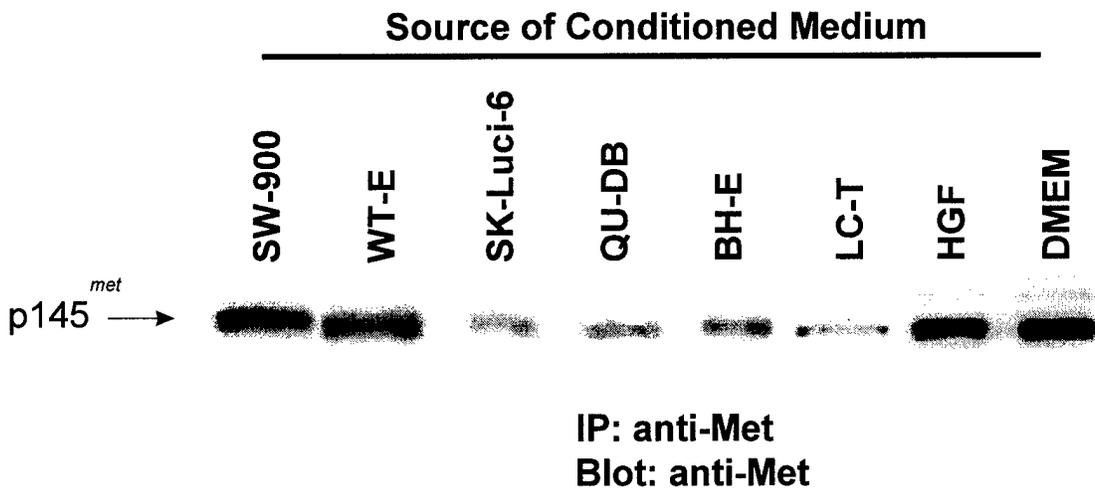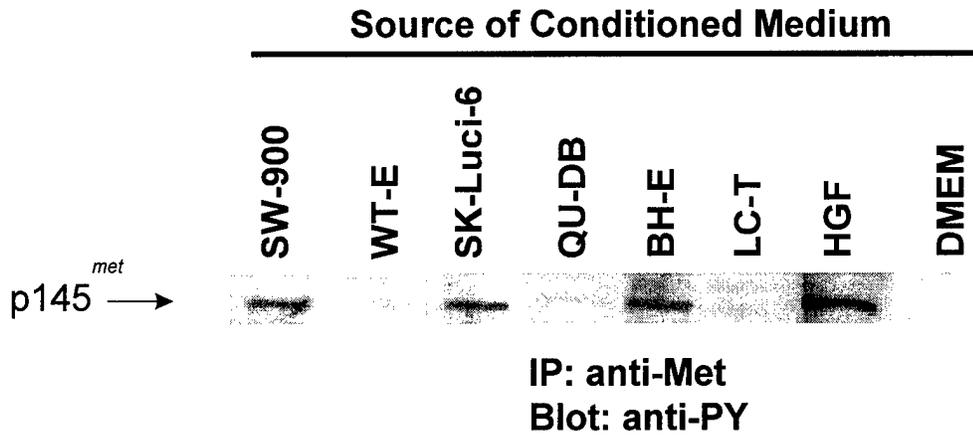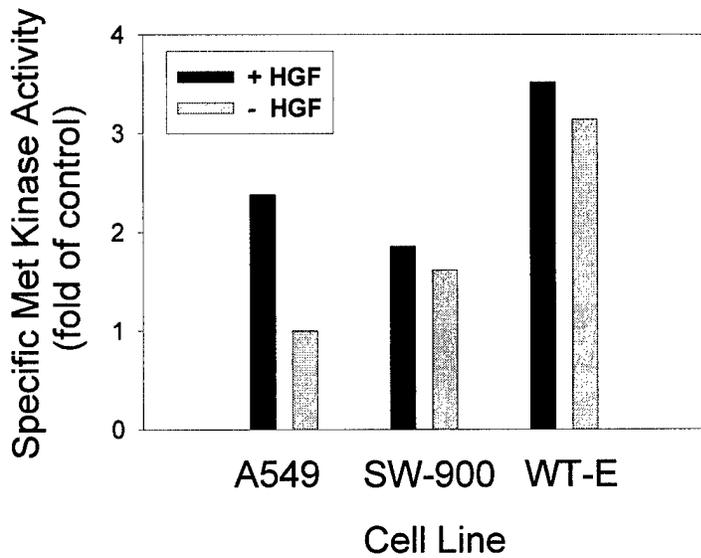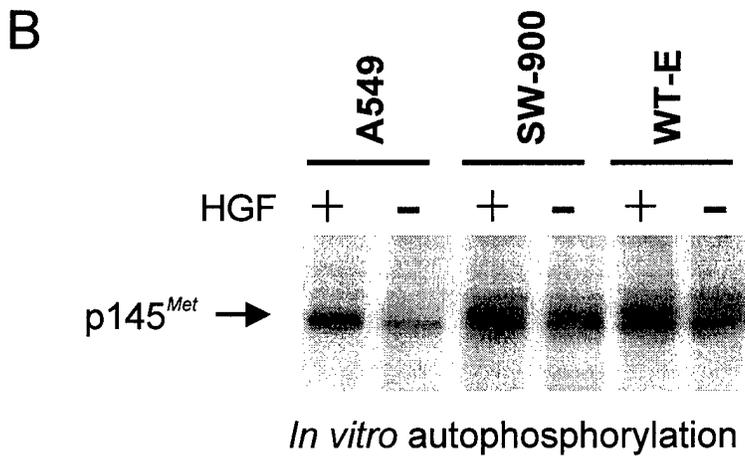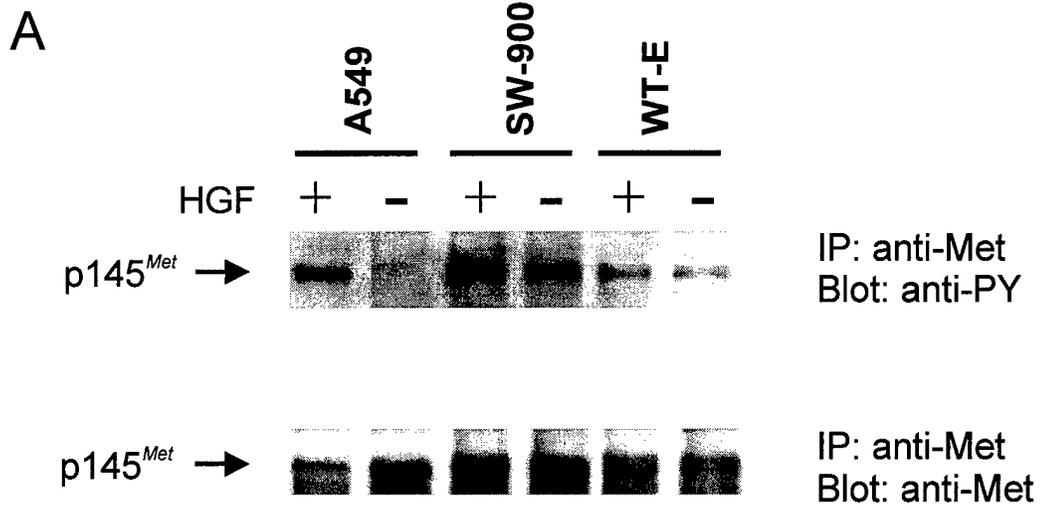
Figure 5

A

B

Figure 6

Figure 7

Blot: anti-p-ERK1/2

Blot: anti-ERK2